Biotechnological approaches for enhancement of bioactives in tubers of *Decalepis hamiltonii*

Final Synopsis

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Introduction

Growth in food industry runs hand-in-hand with the growth in additive requirement, one such additive is a flavor that ranks first, both in usage and market. Among all the flavour compounds, vanillin flavor tops first with global market value of approximately $600 million, only a small fraction of this volume consists of natural vanilla, with the vast majority being synthetic vanillin (http://www.etcgroup.org/sites/www.etcgroup.org/files/Vanilla) having over 97% of overall global demand. Natural flavors usage is ~ 80% in beverages, 20% in sweets and candies. Natural vanillin market size (derived from Vanilla pods) represents less than 1% of the overall industry size and was over 60 tons in 2015. Besides vanillin, there are wide range of fruit and flower-based flavours; one such tuber based flavour molecule that is not much explored is 2-hydroxy-4-methoxy benzaldehyde (2H4MB), an isomer of vanillin reported in *Decalepis hamiltonii* Wight & Arn., belongs to Asclepiadaceae (Sharma and Shahzad 2014). *D. hamiltonii* is an endemic twiner having natural habitats confined to the Deccan peninsula and Western Ghats of South India. Traditionally, its tuberous roots find a place as a local delicacy in various recipes as culinary spice. Also it is widely used as an appetizer, blood purifier and preservative in culinary recipes. Besides, studies reporting the traditional uses of the tuberous root and its extracts as insecticidal, antifungal and antioxidant are available (reviewed by Pradeep et al., 2016). The tubers of this endangered plant highly exploited (medicinal and food applications) as a substitute for Indian sarsaparilla (*Hemidesmus indicus*) through destructive harvesting methods, hence, its conservation is crucial.

The unique flavour molecule 2H4MB has similar aroma to vanillin and could be a better alternative for natural vanillin, if it can be produced in large scale to meet the international market. However, this flavour has GRAS (https://www.femaflavor.org /sites/default/files/23.%20GRAS%20Substances%20%284254-4429%29.pdfce) status but not yet industrialized due to lack of enough raw materials and endangered status. Biotechnological approach can be used to produce this flavour naturally in large scale (Ravi Shankar, et al., 2000). Consequently, it is vital to exploit this exotic flavour molecule to the international market without disturbing its habitat. Apart from this major flavor attributing 2H4MB, the tuberous roots of *D. hamiltonii* are reported to be a repository of other bioactive metabolites which are

expected to contribute to its pharmacological functions such as anti-oxidant (Harish, et al., 2010) antidiabetic (Naveen et al., 2010), hepatoprotective (Srivastava, et al., 2010), neuroprotective (Kamireddy et al., 2018) effects etc. Phenolics, flavonoids and sitosterols are present in moderate to very small quantity in the solvent based extractives of tubers (Pradeep et al., 2016) and also in their volatile fraction (Nagarajan and Jagnmohana Rao 2001; Mohan et al., 2016). All these bioactive metabolites have a synergistic activity that contributes to *D. hamiltonii* tuber-extracts biological properties.

The quality and quantity of fatty acids content of the plant parts play vital role in human health (Dorni et al., 2017). Lipids being an important part of the human diet have a critical role in providing the required energy and essential fatty acids (Li eta l., 2005). Furthermore, they are imperative for carrying lipid soluble vitamins, and synthesis of prostaglandins and steroidal hormones (Aktumsek et al., 2011). The volatile fraction metabolites of *D. hamiltonii* tubers mainly constitute 0.68% of its fresh weight from which 2-hydroxy-4-methoxybenzaldehyde crystallized out 96%. (Nagarajan and Jagnmohana Rao 2001). However, oil fatty acid composition of the tuber extracts not known. The downstream processing of bioactives from the plant parts would affect the quality and quantity of these bioactives, so appropriate methods of processing should be aided with enhancement of the desired bioactives in the extracts so that there will not be any compromise in the quality of plant extracts for end-user applications. The same is applicable to this economically important swallow root (*D. hamiltonii*), where in bioactives potentiation is having greater importance from functional food applications point of view. However, in *D. hamiltonii,* enhancement of bioactives, sustainable methods for its propagation and effective & ecofriendly downstream processing of bioactive fractions received scant attention.

Today, a number of root extract fortified or added products are developed which are having good antioxidant potential and other health benefits. Such plant based products have a great market in the global market and are utilized for management of various diseases. Recently, CSIR-CFTRI had developed a solvent-free flavor essence from the tuber extracts of *D. hamiltonii* for wide range of food applications (https://cftri.res.in/technologies/SFS/fed.pdf). In view of recent increase in the awareness for natural flavor extracts from this plant and also due to huge interest on its commercial cultivation by the progressive farmers in parts of the Karnataka state, there is a scope for looking at unexplored R&D avenues of *D. hamiltonii*.

Accordingly, the following are the objectives of the thesis work:

Objectives

1. To enhance bioactives in tubers of *D. hamiltonii* in *in vitro* and *ex vitro.*
2. To develop *in vitro* tuberization and evaluation of its bioactives *vis-à-vis ex vitro* tubers.
3. To optimize the methods for potentiation of bioactives of root extracts.

Organization of the thesis

The first and second chapter of the thesis comprises the introduction and review of the literature of the research subject respectively, followed by three chapters of work carried out towards the objectives in sequential order titled appropriately as in the section below and in the end summary and conclusion of the thesis work provided.

Chapter 1: General Introduction

This chapter provides a brief introduction of *Decalepis hamiltonii* plant, its natural habitat, general features, traditional applications in food and medicine, important areas of research pertain to this plant that carried out. This chapter also explains the definition of the problem and purpose of the study.

Chapter 2: Review of Literature

The second chapter reviews the origin, botanical and taxonomical description of *Decalepis* and the major differences among the species of *Decalepis*. It briefly describes various important bio-actives of *D. hamiltonii* with special mention on the phenylpropanoid pathway metabolites, phenolics and flavonoids. The chapter compiles literature on the phytochemicals, biochemical leads on the flavor metabolites biosynthetic pathway, and preliminary leads on proteomics. Apart from this, a brief overview of a hypothetical representation of the PPP based vanillin flavor biosynthetic pathway was given. Also, information on *in vitro* cultivation methods and biotechnological approaches for the sustainable propagation, and conservation of *D.hamiltonii* was briefed. This chapter culminates with the prior art on improving its biaoctives, mentioning the necessity of various post-harvest extraction of bioactives from *D. hamiltonii* tubers for value addition.

Chapter 3: To enhance bioactives in tubers of *D. hamiltonii* in *in vitro* and *ex vitro.*

This chapters covers the first objective of the thesis work wherein, the influence of elicitation on *in vitro* callus suspension cultures biomass, and 2H4MB, TPC and TFC content was studied. Also, elicitor mediated improvement of *ex vitro* seedling plant tubers growth and

their bioactives were recorded. In callus cultures maximum content of 2H4MB production 0.079 ± 0.01 mg $100g^{-1}$ DW and biomass 197.5±1.5gL^{-1} were observed by 4^{th} week of culturing. Elicitation treatment to suspension cultures by *m*-topolin (15 μM), pectin (15 μM) and SNP (10 μM) supported 0.31 $mg100g^{-1}$ DW, 0.27 $mg100g^{-1}$ DW and 0.21 $mg100g^{-1}$ of 2H4MB production respectively by 4th week. Immobilization of *D. hamiltonii* callus cells in calcium alginate matrix influences 2H4MB production. In 3% and above concentration of alginate beads, 2H4MB was observed to be in the immobilized within alginate bead. But at lower concentration of alginate beads it was observed to be leached out into the culture medium. However, 2H4MB was recorded to be two-folds higher in beads which are made of low percent of alginate. Attempts were made to scale up the callus biomass and metabolites production in stirred tank bioreactor (STB) containing 2 lit culture medium. A 1.69 fold increase in biomass and 0.024± 0.007 mg $100g^{-1}$ DW 2H4MB upon 7 days growth was recorded. The methanol extract of fermenter-grown-callus showed 2.13± 0.39 mg $100g^{-1}$ DW and 0.12± 0.4 mg $100g^{-1}$ DW of TPC and TFC respectively.

Simultaneously, 3 month old seedling plants in greenhouse were subjected to elicitation by different concentrations of Cu–chitosan nanoparticle. Cu–chitosan Nano particle at 40 ppm concentration showed best response for plant growth. Upon completion of one year growth in pots, the plants produced 137 ± 4.85 g FW of tuber biomass and 1.124± 0.04 mg $100g^{-1}$ DW 2H4MB, compared to 105 ± 2.5 g FW of tuber biomass and 0.721± 0.01 mg $100g^{-1}$ DW of control plants.

The study infers that the elicitation improves 2H4MB content in callus suspension cultures of *D. hamiltonii*. It was observed that *D. hamiltonii* callus culture in a bioreactor can be an alternative method for producing vanillin isomer in large-scale production and further optimization of culture conditions in this regard is required. Cu–chitosan Nano particle was observed to enhance bioactives in tubers of *D. hamiltonii*.

Chapter 4: To develop *in vitro* tuberization and evaluation of its bioactives *vis-à-vis ex vitro* tubers.

The second objective of the thesis work that covered in this chapter contains three subcomponents. The first subcomponent pertains to the induction of *in vitro* rooting under nitrate stress in culture medium followed by their hardening and *ex vitro* performance evaluation in pots for the next one year. The tuber biomass, and bioactive content such as 2H4MB, TPC and TFC

were compared with that of *ex vitro* raised seedling plants growth features. The effect of different nitrate concentrations in MS medium for *in vitro rooting* revealed positive at ¼ and ½ nitrate strength by 14 d inoculation. The *in vitro* rooted plants (IVP) showed 80% survival upon hardening and exhibited similar growth pattern to seedling plants. The IVP grown from ¼ nitrate strength media produced 155 ± 4.85 g FW of tuber biomass which is observed to be higher than full nitrate strength IVP (112 ± 2.52 g) which are grown at similar conditions in the greenhouse. The flavor metabolite content, total phenolic, total flavonoids, and antioxidant potential of these IVP tubers were superior to that of *ex vitro* seedling plants. Upregulation of flavor biosynthetic pathway genes *DhPAL, DhC4H* and *DhCoMT* were observed in tubers of potted plants that developed from low nitrate strength culture media.

Another sub-component of this objective is to record the tuber formation from the seedling plants *ex vitro* under limited supply of pot soil. Pots holding 125cc soil showed a phenomenal variation in tuber size and shape, where in round and short tuber was noticed after 3 months followed by its further enlargement in size and tuber buds' formation. Seedling plants allowed to grow in presence of higher quantity of soil (375 cc) in pots, supported conical to long tubers which is a normal tuber shape in *D. hamiltonii.*

Yet another sub-component (third) is to trigger tuberization from *D. hamiltonii in vitro* shoots and *in vitro* raised seedling shoot explants wherein 5-10% and 15-20% of explants responded for meager to moderate rooting along with sporadic micro tubers induction on WPM medium supplemented with BA and NAA. The best response was observed in presence of 4 mgL^{-1} BA and 3 mg L^{-1} NAA containing medium by producing micro tubers i.e. 1-2 no. & 3-5 no. from *in vitro* shoot explants and seedling shoot explants respectively. However, upon hardening of these plants in greenhouse, the percent survival was poor (10%).

This study has significance since it helps to establish potted plants with good mini tuberous roots and upon their field transfer it may be helpful to the plant to accustom well to the field environment and to pick up fast growth.

Chapter 5: To optimize the methods for potentiation of bioactives of root extracts.

In this chapter, the work component belongs to the fatty acid profile of *D. hamiltonii* tubers, immobilization of *in vitro* callus biomass of *D. hamiltonii* by using alginate, and encapsulation studies using tuber extracts by spray-drying and freeze drying.

The fatty acid profile of *D. hamiltonii* tubers total oil was characterized in maturation of tubers. The oil content of these tubers was in the rage of 3–4% which contains saturated fatty acid (SFA), Mono unsaturated fatty acid (MUFA), poly unsaturated fatty acid (PUFA) in the following percent 59.74%, 20.71% and 19.51% respectively. The tuber oil is rich in saturated fatty acids, particularly palmitic acid (C16:0) 32.61% followed by other fatty acids steric acid (21.36%) oleic acid (19.08%) and linolenic acid (19.51%). Lipidome analysis of tuber using high-resolution mass spectrometry showed that complete profile of triacylglycerol (TAG) species.

The aqueous flavour extract from tubers of *D. hamiltonii* was encapsulated by spray drying and freeze-drying. Carrier materials viz., sodium caseinate, maltodextrin and Gum acacia were used alone and also as blends to understand their collective effect during encapsulation. Among the carrier materials used sodium caseinate showed good retention of bioactives and flavor (2H4MB) which was quantified by HPLC (encapsulation efficiency 82% and yield 40% ww^{-1}) and confirmed by ^{1}H NMR. However, in this study considering flavor-retention and powder-yield (encapsulation efficiency 74% and 54% ww^{-1}), maltodextrin in combination with sodium caseinate (MS) was observed to be the best carrier material for spray drying. These "MS" microcapsules are stable and showed 70% retention of flavor after 3 months storage at room temperature and the microbial load was found within in the limits. Since spray dry based microencapsulation is continuous and cost-effective the same can be employed for *D. hamiltonii* bioactives for food and nutraceutical applications.

Fatty acid and lipidomic profile of *D. hamiltonii* tubers was carried out. Immobilization of callus cultures using calcium alginate seems to be promising in improving the 2H4MB content. The bioactives in aqueous extract of tubers was encapsulated by spray drying and freeze drying for food applications.

Chapter 6: Summary and conclusion

1. Augmented production of vanillin flavor metabolite 2H4MB was achieved by challenging the *D. hamiltonii* callus suspension with m-topolin, pectin and sodium nitroprusside.
2. A substantial increase of 2H4MB i.e. 4.5 folds under m-topolin elicitation, followed by 3.6, 2.4 folds in presence of pectin and sodium nitroprusside was recorded.

3. It was observed that, callus immobilized in calcium alginate beads (3%) has shown more accumulation of 2H4MB, however at low concentration of (< 3%) alginate treatment, more content of flavor metabolite 2H4MB found in the culture medium.
4. Preliminary studies to find the suitability of stirred tank bioreactor (STB) for callus biomass scale up indicates a 1.69 fold increase in biomass and 0.024± 0.007 mg $100g^{-1}$ DW 2H4MB upon 7 days growth. The methanol extract of this bioreactor-grown-callus showed 2.13± 0.39 mg $100g^{-1}$ DW and 0.12± 0.4 mg $100g^{-1}$ DW of total phenolic content (TPC) and total flavonoid content (TFC) respectively.
5. Cu–chitosan nanoparticle application to *D. hamiltonii* plantlets has shown enhancement in production of 2H4MB, in *ex vitro* plants with substantial increase in 70-75% tuber biomass yield and 60-65% increase the production of 2H4MB.
6. Upon hardening 80% survival observed for the *in vitro* rooted plants (IVP) and their growth pattern on par with the seedling plants. The IVP grown from ¼ nitrate strength media produced 155 ± 4.85 g FW of tuber biomass which is observed to be higher than full nitrate strength IVP (112 ± 2.52 g) which are grown at similar conditions in the greenhouse.
7. The flavor metabolite content, total phenolics, total flavonoids, and antioxidant potential of these IVP tubers were superior to that of *ex-vitro* seedling plants.
8. The pots containing 125cc soil mix showed a phenomenal variation in tuber size and shape, where in round and short tuber was noticed after 3 months followed by its further enlargement in size and tuber buds formation. However, at higher volume of soil (375 cc) in pots, allowed the formation of long tubers.
9. The HPLC analysis of the methanolic extracts of the these tubers reveals moderately high content of flavor compound 2H4MB, TPC and TFC compared to that of plants grown in pots containing 375 cc soil mix.
10. Induction of *in vitro* tuberization from *D. hamiltonii* micro-shoots was accomplished, however the percentage of explants responded for the same is less and recorded as 5-10% and 15-20% for *in vitro* shoots and in vitro raised seedling shoot explants.
11. The rooting from the explants was found to be meager to moderate along with sporadic micro tubers induction on WPM medium supplemented with BA and NAA.

12. The best response for rooting and *in vitro* tuber induction was observed in presence of 4mgL^{-1} BA and 3 mgL^{-1} NAA containing medium by producing 1-2 no. & 3-5 no. micro tubers from *in vitro* shoot explants and seedling shoot explants respectively.
13. The oil obtained from the tubers (3-4%) of *D. hamiltonii* was observed to be highly viscous due to its latex. The percentage of saturated fatty acid (SFA), mono unsaturated fatty acid (MUFA), poly unsaturated fatty acid (PUFA) was observed to be 39.51%, 17.02% and 43.69% respectively in fresh matured of tubers harvested from 5 years old filed grown plant.
14. Among the fatty acids composition palmitic acid (C16:0) was observed to be predominant (27.1%) followed by steric acid (11.16%).
15. The other major fatty acids which constitute *D.hamiltonii* tuber oil include oleic acid (16.89%) and linolenic acid (10.19%).
16. The aqueous extract of *D. hamiltonii* tubers contain 2H4MB (5.3±0.1 mg 100ml^{-1}), TPC (27.4±0.5 mg 100ml^{-1}), and TFC (13.7±0.2 mg 100ml^{-1}) and the same was used for spray drying and freeze drying studies.
17. In encapsulation studies three carrier materials viz., sodium caseinate, maltodextrin and Gum acacia were used alone and also as blends, sodium caseinate showed good retention of bioactives and flavor (2H4MB) which was quantified by HPLC (encapsulation efficiency 82% and yield 40% ww^{-1}) and confirmed by ^{1}H NMR.
18. However, in this study considering flavor-retention and powder-yield (2H4MB encapsulation efficiency 74% and 54% ww^{-1}), maltodextrin in combination with sodium caseinate (MS) was observed to be the best carrier material for spray drying.
19. The combination of maltodextrin and sodium caseinate microcapsules are found to be stable and displayed 70% holding of flavor after 3 months storage at room temperature and the microbial load was found within in the limits.
20. In freeze drying too, three carrier materials viz., sodium caseinate, maltodextrin and Gum acacia were used alone and also as blends. Sodium caseinate showed good retention of bioactives and flavor (2H4MB) which was quantified by HPLC (encapsulation efficiency 84% and yield 97% ww^{-1}).
21. The combination of maltodextrin and sodium caseinate showed 41% retention of flavor after 3 months storage at room temperature.

Conclusion

The callus suspension culture in a bioreactor can be an alternative method for producing vanillin flavor metabolite 2H4MB and other bioactive. *In vitro* rooting under low nitrates comprising culture medium has substantial and sustainable influence on quality parameters of tubers. The nitrate concentration in culture medium has significantly influenced the *in vitro* rooting of the micro shoots. It has reduced 1/3rd time in initial rooting and the overall plant regeneration which is very crucial for *in vitro* propagation of this endangered plant. Improved tuberization from seedling explants with varied shape was evident under low quantity of soil in pots *ex vitro*. During the encapsulation studies it was observed that milk-based protein i.e. sodium caseinate as carrier material showed good efficiency and blending with gum-acacia or maltodextrin has displayed synergistic effect. Since spray dry based microencapsulation is continuous and cost-effective, the same can be employed for *D. hamiltonii* bioactives for food and nutraceutical applications. Natural flavor extract powder with an antioxidant potential from swallow root is a new value-added product, which can be used in multiple food formulations.

Future studies

The response for improved growth parameters of tubers, flavor and other bioactives obtained in this study has to be further substantiated by employing molecular methods. The preliminary leads obtained in bioreactor based biomass culturing, needs further validation by optimizing culture conditions and by advocating elicitors and growth-promoting agents for scale up. Also, the encapsulated flavor rich powder could be further explored as a natural flavor-additive in a wide range of food products.

List of publications:

- Koppada, Umashankar, Pradeep Matam, and Giridhar Parvatam. "*In vitro*-derived plants grown from low nitrate medium produced quality tubers in *Decalepis hamiltonii* Wight & Arn." 3 Biotech 12.9 (2022): 1-11.
- Koppada U, Matam P, Parvatam G *In vitro* elicitation supports the enrichment of 2H4MB production in callus suspension cultures of *D. hamiltonii* Wight & Arn Rom Biotechnol Lett . 2022; 2 7 1): 33023308. DOI: 10.25083/rbl/2 7133023308
- K. Uma Shankar, P. Giridhar Biomass production and bioactive secondary metabolites of bioreactor grown callus suspension cultures of Swallow root (*Decalepis hamiltonii*

Wight & Arn.) Journal of Emerging Technologies and Innovative Research 2022, Volume 9, Issue 3, Page: g153-g158

Conference/ Workshop / Poster

- Uma Shankar 8th International food Convention #IFCoN 2018 organized by AFSTI during 12 - 15 December at CSIR-CFTRI, Mysore, India.
- Participated in NER twinning program & Fold scope seminar Sponsored by Department of Biotechnology on 19th February 2019, at CSIR-CFTRI, Mysore.
- Uma Shankar et al. "Encapsulation of vanillin-flavour compounds from tubers of endangered woody climber: *Decalepis hamiltonii*" 7th "BIOPROCESSING INDIA 2019" 14-16 December, 2019 Mysore, India

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